Modifications of 60 S ribosomal subunits induced by the ricin A chain

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Incubation of 60 S ribosomal subunits with the ricin A chain reduced their stability during heat treatment. The toxin shifted the thermal denaturation curve of the subunits towards lower temperatures, in a similar way to that produced by the decrease in Mg^{2+} concentration. A brief heating (3 min at 57°C), which did not affect control subunit activity, enhanced protein synthesis inhibition of the toxin-treated subunits that released more 5 S RNA, in the form of nucleoprotein complex(es) with protein L5 and phosphoproteins P1P2 (RNP_H), than did heated control subunits [(1984) Eur. J. Biochem, 143, 303–307]. No nuclease activity tested on 60 S subunits and purified 5 S and 5.8 S RNA was found associated with the toxin. The results suggest that the toxin induced a limited conformational change of the 60 S subunit, which destabilized the interaction between RNP_H and the rest of the subunit.

Ricin (Eucaryote) Ribosomal subunit RNase 5 S RNA

1. INTRODUCTION

The ricin A chain inactivates the eukaryotic 80 S ribosomes by damaging their 60 S subunits. It inhibits the EF-2 dependent GTPase and the proteosynthetic activities of these subunits (see [1]). Inactivation of damaged subunits can be largely overcome by increasing the concentrations of EF-2 and Mg^{2+} [2,3]. It has therefore been suggested that the ricin A chain might cause a conformational change in 60 S subunits that would restrict the accessibility of the subunits to the EF-2-GTP complex [3]. In order to test this hypothesis we analyzed the influence of the toxin on the thermal denaturation profile of the 60 S subunits, which is a sensitive way of detecting small conformational changes in ribosomes [4,5]. In connection with this study we found that a brief heat treatment revealed modifications induced by the toxin. While this paper was in preparation, the ricin A chain was reported to possess a high nuclease activity [6] in contradiction with previous studies, in which neither nuclease nor protease activities had been found associated with the toxin [7,8]. We therefore carefully examined our preparation for any trace of nuclease activity.

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2. EXPERIMENTAL

2.1. *Materials*

Purified ricin A chain was a gift from Dr P. Gros of Sanofi laboratories. It gave a single band on dodecyl sulfate-gel electrophoresis. In some experiments it was labelled by reductive methylation using ^3H-labelled $NaBH_4$ (185 GBq/mmol, from the Commissariat à l'Energie Atomique). The specific activity of ^3H-labelled protein was 467 kBq/mg. α-Sarcin was a gift of Dr Vasquez. 95% pure EF-1 (from reticulocytes) and EF-2 (from calf brain) were gifts from Dr Y. Cenatiempo and A. Parmeggiani. 60 S subunits were prepared from rat liver as previously described

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[10]. Small rRNAs extracted from intact yeast cells (*Saccharomyces cerevisiae*) with phenol were separated into 5 S and 5.8 S RNA species by two successive electrophoreses in 7 M urea (10 and 15% polyacrylamide) using 50 mM Tris borate, pH 8.3, 1 mM EDTA and then extracted from the gel. Caution was taken throughout to use sterilized labware and water, and RNase-free reagents.

2.2. *Ricin treatment of 60 S subunits*

344 pmol of subunits were incubated with or without 0.8 molar equivalent of toxin in 175 μl of 1 mM potassium phosphate, pH 7.4, 30 mM KCl, 5 mM $MgCl_2$, 6 mM 2-mercaptoethanol (buffer A) for 10 min at 37°C. One part of each sample (60 S(RA) or 60 S(C), respectively) was used for melting studies. The remainder was divided into two parts, one of which was heated 3 min at 57°C; both were used for polyphenylalanine synthesizing assays and RNA analyses.

rRNAs extracted from 60 S(RA) subunits with phenol were analyzed on two-dimensional gel electrophoresis using a 2.5–10% polyacrylamide concentration gradient in the first dimension, 5% polyacrylamide-7 M urea in the second dimension and the Tris borate buffer described above.

The 5 S RNA protein complex (RNP_H) released from 60 S(RA) and 60 S(C) subunits heated at 57°C was analyzed on two-dimensional gel electrophoresis. The first-dimension gel was as described in [11] except that we used a 2.4–7.5% polyacrylamide concentration gradient. The second-dimension gel in 12% polyacrylamide included 0.2% SDS and 6 M urea, in order to resolve the RNA and protein components of RNP_H. For identification all the protein spots of the RNP_H complex (already identified in two-dimensional gel electrophoresis [11]) were extracted separately from slabs stained with Coomassie blue, and analyzed in two-dimensional gel electrophoresis (acidic-SDS) system [12].

2.3. *Ricin treatment of purified RNA*

Yeast 5 S or 5.8 S RNA were incubated with either the ricin A chain or α-sarcin in 44 mM potassium phosphate, pH 7.4, 0.4 mM EDTA, 7 mM 2-mercaptoethanol as noted in fig.3 and extracted with phenol. After ethanol precipitation RNA was heated (65°C/3 min) in 7 M urea, 4 mM Tris borate, pH 8.3, 0.1 mM EDTA, and quickly chilled at 4°C. One part was analyzed by one-dimensional gel electrophoresis in 10% polyacrylamide-7 M urea and visualized with ethidium bromide. The remainder was precipitated with ethanol, 3′-end labelled at 4°C/16 h with [5′-^{32}P]pCp (111×10^3 Gbq/mmol from Amersham), T_4 RNA ligase and ATP [12], precipitated with ethanol and electrophoresed as described above. Autoradiography was performed using X-OMAT Kodak films.

3. RESULTS

In three different experiments, the whole thermal denaturation curve of 60 S(RA) subunits dialyzed against buffer A containing 5 mM $MgCl_2$ as compared with that of 60 S(C) significantly shifted towards lower temperature (fig.1a,b). The lowering of Mg^{2+} concentration down to 0.6 mM $MgCl_2$ did not modify the melting-out curve of 60 S(RA) but shifted that of 60 S(C) almost to the position of the 60 S(RA) curve (not shown). Total

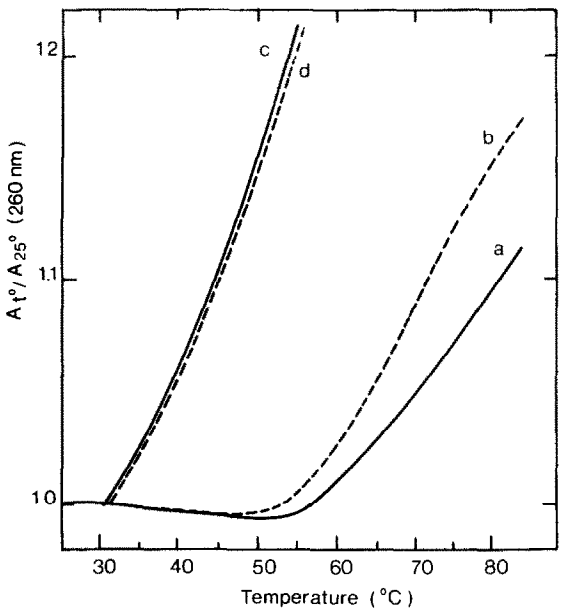

Fig.1. Melting-out curves of the ricin A chain-treated subunits and their rRNAs. Control 60 S subunits (a), subunits incubated with the ricin A chain (b) and total rRNAs extracted from them (c and d, respectively) were heated in buffer A at the rate of 1°C/min. Absorbance at 260 nm was recorded using a Gilford spectrophotometer equipped with a thermoprogrammer.

rRNAs extracted from 60 S(RA) and 60 S(C) showed identical melting-out curves (fig.1c,d).

Data listed in table 1 show that the extent of protein synthesis inhibition brought about by the ricin A chain in 60 S subunit, under our conditions, was about 60% when assayed in 6 mM $MgCl_2$. This inhibition decreased when we used a higher concentration of $MgCl_2$ in the assay (10 mM), which confirms other studies [3]. The extent of protein synthesis inhibition induced by the toxin increased by a factor of about 3 when 60 S(RA) subunits were briefly heated prior to being assayed at 10 mM $MgCl_2$. In contrast with this, 60 S(C) subunits heated under the same conditions retained their initial activity almost completely. The fact that the 60 S subunits used here contained about 0.5 molecule of tRNA might explain the inability of the ricin A chain to inactivate these more completely, even after heating at 57°C. Indeed, tRNA binding inhibits the ricin A chain binding to rat liver ribosomes and protects them from inactivation by the toxin [3]. This tRNA is not removed by the heat treatment [14]. The presence of bound tRNA probably explains the variability in the extent to which ricin A chain can inactivate subunits from different preparations, a perplexing feature frequently reported [15].

Table 1

Effect of Mg^{2+} concentration and heat treatment on poly(U) translation activity of the ricin A chain-treated 60 S subunit

Material	$[Mg^{2+}]$ (mM)	Residual activity of polyphenylalanine synthesis (% of control)
60 S(RA)	6	42
60 S(RA)	10	78
60 S(C)[a]	10	95
60 S(RA)[a]	10	40

[a] Heated 3 min at 57°C prior to activity measurement

The activities are expressed as percentages of the activities of control subunits incubated 10 min at 37°C without the ricin A chain. The 100% values corresponded to 11 and 15 pmol of [^{14}C]phenylalanine incorporated when polyphenylalanine synthesis was measured in 6 mM and 10 mM $MgCl_2$, respectively, using 2.5 pmol of 60 S, an equimolar amount of 40 S, 2.4 μg of EF-1, 0.6 μg of EF-2 for a final incubation mixture of 125 μl as described [10].

We have recently reported that a ribonucleoprotein complex (RNP_H) containing 5 S RNA and a small number of proteins, mainly L5 and the acidic phosphoproteins P1P2, is released from heated 60 S subunits. This complex was isolated by 1D-gel electrophoresis and by sucrose gradient [11]. Since the ricin A chain treatment increased the thermolability of 60 S subunits, it was interesting to compare the amount and composition of the RNP_H complex released from 60 S(RA) subunits and 60 S(C) subunits. In these experiments we used a 2D-gel electrophoresis method that allows fractionation, with a good resolution, of all the products derived from heated subunits in the first dimension, and analyses the components of these products in the second dimension (fig.2). Thus the electrophoretogram of control subunits heated for 3 min at 57°C showed that most of the released 5 S RNA moved in the first dimension slower than free 5 S RNA, as a complex with L5, which in the second dimension was resolved into two spots as already reported [19,20], and P1P2. A close examination of the electrophoretogram suggests, in fact, a heterogeneity of the complex: part of the 5 S RNA ran with L5 and part of it with P1P2, slightly to the left of L5. Free RNA fragments of molecular mass close to 100 kDa, which moved separately from the RNP_H complex in 7.5% acrylamide (first dimension), were also visible on the electrophoretogram. The pattern of 60 S(RA) subunits heated to 57°C showed two differences with the pattern described above. First, the amount of RNP_H as measured by its content in 5 S RNA and protein components, was always strongly increased (about 3-fold); the continuous streaks presumably resulted from slow breakdown of the complex during first-dimension electrophoresis. Second, protein P3, which is a well-defined derivative of P2 [21], was observed under P1P2. This only occurred when P3 was already present in the starting 60 S subunits, which was not always the case. The reason why P3 was not observed in the RNP_H released from control subunits has not so far been explained. The possibility that modifications of the electrophoretogram in the P3 area were due to artefactual degradation products of the ricin A chain was excluded, since no radioactivity was found at this place when the 60 S

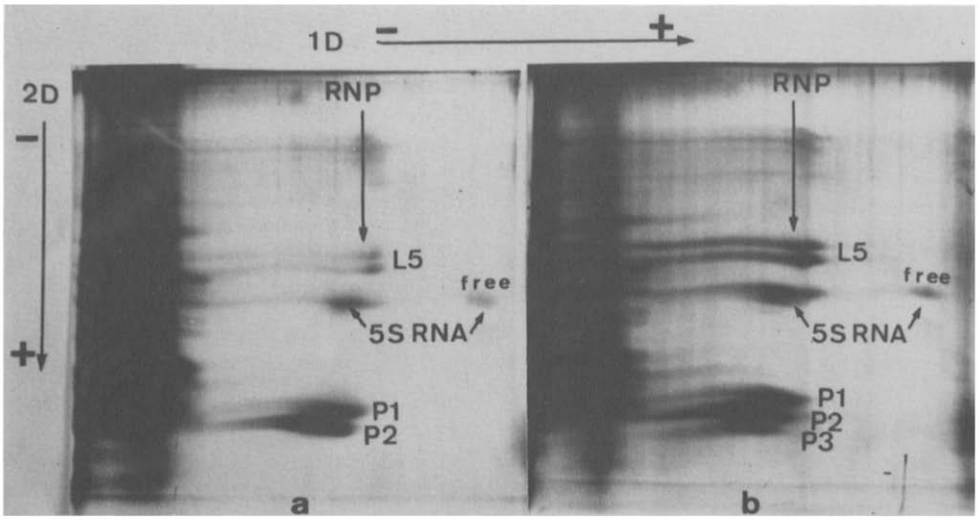

Fig.2. Effect of the ricin A chain on RNP_H release from 60 S subunits. 60 S subunits (100 pmol) incubated for 10 min at 37°C without or with the ricin A chain and then heated for 3 min at 57°C (a and b, respectively) were electrophoresed in two-dimensional gels as described in section 2. The spots of RNA and of proteins were successively stained by silver, using a method adapted from [16] and [17] (Paleologue, A., unpublished). They were quantified by a method adapted from [18] by eluting the silver grains from the photographs with 1 M NaOH and measuring the absorbance of the eluate. Free 5 S RNA was identified by using purified 5 S RNA in control gel electrophoresis.

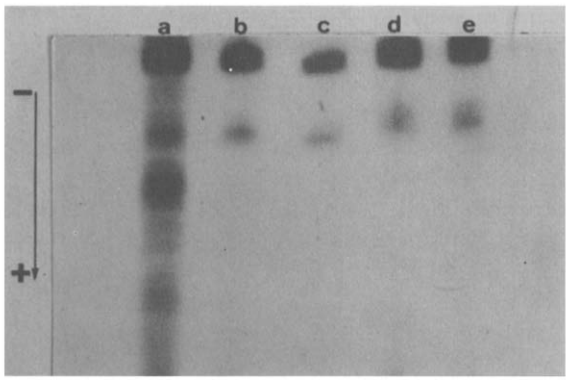

Fig.3. Ribonuclease activity assay of the ricin A chain and α-sarcin. Purified 5 S RNA was incubated (37°C/10 min) with 0.7, 4, 25 molar equivalents of the ricin A chain (c, d and e, respectively), 10 molar equivalents of α-sarcin (a) or without toxin (b) (see section 2). After phenol extraction 1.35 pmol aliquots were 3'-end labelled and electrophoresed according to Peattie [13] (500 V for 1 h).

subunits were incubated with labelled A chain.

The 2D-gel electrophoresis patterns of rRNAs extracted from 60 S(RA) subunits, heated or not, were always identical to those obtained for rRNAs from control subunits, which suggested that ricin had no nuclease activity, at least on 60 S subunits (not shown). In other experiments purified 5 S and 5.8 S RNAs were incubated with increasing amounts of the ricin A chain up to a 25 molar ratio of toxin/RNA which is 31 times the ratio used in our experiments with 60 S subunits. The resulting RNA products, 3'-end labelled or not, were analyzed by 1D-gel electrophoresis under denaturing conditions (see section 2) and either autoradiographed or revealed by ethidium bromide. In 4 different experiments, no RNA degradation was observed whatever the ricin concentration used. Fig.3 illustrates the results of 5 S RNA incubation followed by 3'-end labelling: ricin A did not create new 3'-ends. On the contrary α-sarcin, a toxin known to possess a ribonuclease activity [22] and which was used here at a toxin/5 S RNA ratio of 10, did create many new 3'-ends.

4. DISCUSSION

From all the results reported in this study, it can be concluded that the ricin A chain induces a limited conformational change of the 60 S ribosomal subunits. The modifications of the thermal denaturation pattern of the subunit treated with the ricin A chain suggest that the toxin affects non-covalent interactions between ribosomal proteins and rRNAs. Ricin treatment reduces subunit heat stability, just as when subunits are equilibrated with a low Mg^{2+} buffer, and should slightly unfold the subunit. This unfolding could explain the observation that the inhibiting effect of the toxin on poly(U) translation activity is partially reversed by using high Mg^{2+} concentrations in the assay (see table 1 and [3]).

Our observation that, after a brief heat treatment, toxin-treated subunits released more RNP_H complex than control subunits suggests that the toxin weakens hydrogen bonds which maintain this complex attached to the subunit. The stronger inactivation of the ricin-treated subunit after heating can be explained by the larger RNP_H release. We have recently shown that the RNP_H complex isolated on sucrose gradient as described in [11] possesses some EF-2-dependent GTPase activity (Reboud, A.M., unpublished) as does the 5 S RNA-L5 complex [23]. The action of ricin, which modifies EF-2 binding to ribosomes and the associated GTPase activity could therefore be related to the effect of the toxin on this complex. In any case, the damage induced by the ricin A chain appears to be limited, since the 5.8 S RNA localized at the ribosomal interface [24], probably close to 5 S [25] and hydrogen bonded to 28 S RNA, was not released even after heating the ricin-treated subunits at 57°C.

The problem of whether the limited conformational change induced by the ricin A chain is the primary effect of the toxin or a secondary effect consecutive to a nucleolytic action was examined. Like Mitchell et al. [7] and having used several methods, we did not find any nuclease activity associated within the ricin A chain. The discrepancy between our result and that of Obrig et al. [6], who found that RNA treated with ricin A and 3′-end labelled in the presence of the toxin gave a different pattern from the control and deduced a nuclease action of ricin, might be explained either by a contaminating RNase moiety or by the difference in the experimental procedures: we observed that high amounts of toxin in the labelling mixture hindered RNA labelling and therefore we carried out this labelling after a phenol extraction. It should be stressed that a covalent modification of ribosomal subunit by ricin A would not agree with other findings confirmed by us, i.e. that inactivation by the toxin is overcome by high EF-2 concentration and by toxin removal by centrifugation [15].

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